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Mammary Tumor Development in Mice

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13. ABSTRACT (Maximum 200 Words) We have been using genetically predisposed mice to identify genes that can modify the risk of mammary tumor development. We have found that a congenic strain of mice that carries as a transgene a retroviral insertion of a <i>LacZ-neo<sup>R</sup></i> gene (ROSA26 mice) on chromosome 6, are resistant to <i>Min</i> -induced mammary tumor development. In the ROSA26 mice, the insertion is flanked by about 25 cM of DNA derived from the 129 strain. In order to localize the locus or loci encoding the resistance, we produced mice carrying subsets of the 129 derived DNA from the congenic region. Analysis of six lines indicated that a strong modifier of mammary tumor development mapped within 2 cM of the ROSA26 insertion. This modifier also affects intestinal tumor growth. In addition, prepubertal female and male mice carrying the minimal modifier interval have a significantly smaller extent of mammary gland growth than do age-matched controls. Animals carrying the insertion have slower growth rates than wild-type mice. Finally, we have shown that the effect seen in the ROSA26 mice is due to the insertion and not to linked modifier loci.				
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John K. Allen  
PI - Signature

5/29/01  
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## INTRODUCTION

Progress is being made at identifying genes that are responsible for many cases of hereditary breast cancer. However, the penetrance and time of tumor development can be variable in these families. The effects of background genes on the development of cancer need to be characterized. We propose to use genetically predisposed mice to identify genes that can modify the risk of mammary tumor development. *Apc<sup>Min</sup>* female mice develop mammary tumors with a 5% incidence by 100 days of age. Treatment of female *Min*/+ mice with ENU can increase the incidence of mammary tumors to greater than 80% within 65 days of treatment. We have found that the 129/Sv strain carries alleles that confer resistance to mammary tumors and have identified the chromosomal location of one of the modifier loci. We have identified a congenic strain of mice that carries a 129 allele of the modifier on the B6 background. Using these mice as a starting point, we plan to:

1. Work toward molecular identification of the mammary modifier. By first minimizing the region containing the mammary modifier through genetic recombination, and second, by identifying and testing candidate loci mapping to the region.

2. Test for loss of heterozygosity in the modifier region in mammary tumors.

This project represents a novel approach to the identification of genes controlling breast cancer development. It utilizes the combination of mice predisposed to mammary tumor development and the genetic variability existing in the inbred strains of mice.

## BODY

I have listed below each of the tasks outlined in the Statement of Work from the original grant proposal. After each task, the progress made is described.

### Aim 1:

**Task 1:** Production and identification of 20 male mice carrying recombinant chromosomes. Months 1-12; Mice: 12 ROSA26/+ males, about 50-60 B6 females, 20-30 recombinant males weaned.

**Progress:** The goal of this task is to identify mice carrying recombinant chromosomes to sub-divide the congenic region on chromosome 6. To do this we have crossed B6 females with B6.ROSA26/+ males. The progeny are tested with markers throughout the region to test for the presence of recombinant chromosomes. We have produced and screened over 800 progeny and have identified mice carrying the 6 recombinant chromosomes shown in Figure 1. We expected to identify more recombinant progeny, based on the published map distances between the markers, but have found fewer recombinants than expected. This may indicate that the markers flanking the R26 insertion are closer than indicated in the published maps. However, with the recombinant chromosomes identified, we have been able to minimize the modifier region (see below). Thus, it is unnecessary to identify mice carrying other recombinant chromosomes.

To further characterize the recombinant chromosomes, we have identified 12 PCR-based markers in this region of chromosome 6 that are polymorphic between B6 and 129 mice. These markers have been mapped using the recombinant chromosomes and the DNA from mice from a backcross between 129 and B6. Marker *D6Mit105* is the first marker proximal of the ROSA26 insertion, and *D6Mit55* is the first marker distal to the insertion. The recombination events in Rec1 and Rec2 are between *D6Mit105* and ROSA26. Until further markers are identified, we cannot further define the ends of those recombinant chromosomes. The Rec6 chromosome carries 129-derived DNA at marker *D6Mit55* but not the ROSA26 insertion or *D6Mit11*. *D6Mit11* maps distal to ROSA26 on the mouse maps available from the Jackson Laboratory. We have not been able to separate this marker from ROSA26 (all of the chromosomes that carry the insertion carry a 129 allele at *D6Mit11* and the chromosomes that do not carry the insertion carry B6 alleles), thus we cannot order these markers in our mice.

**Task 2:** Production of female mice carrying each recombinant chromosome. Months 3-18; Mice: 20 recombinant males (produced in Task 1), 120 B6 females, about 200 recombinant females weaned. As recombinant males are identified in Task 1, they will be mated with B6 females to produce the female mice to be used to produce the mice used in Task 3.

**Progress:** The goal of this task is to produce female mice carrying each of the recombinant chromosomes identified in Task 1. These females are needed to cross with *Min*/+ males to produce the mice for testing. Each of the recombinants identified in Task 1 has been crossed with B6 mice to generate the recombinant females. This goal has been accomplished for the all six recombinants identified.

**Task 3:** Production of female *Min*/+ mice carrying the recombinant chromosome. Months 5-24; Mice: 200 recombinant females (from Task 2), about 75 *Min*/+ males, about 800 *Min*/+ females weaned and genotyped for chromosome 6.

**Progress:** Females heterozygous for all six recombinant chromosomes were crossed with B6 *Min*/+ males. The resulting female progeny were genotyped at *Apc* to identify the *Min*/+ females. The *Min*/+ females were then genotyped for markers on chromosome 6. The goal was to identify about 20 *Min*/+ females carrying each recombinant chromosome and 20 of their siblings who carried B6 alleles throughout

the region. Although we have not obtained 20 mice of each genotype for all lines, the statistical analysis indicates that entering more mice into the study will not change the outcome.

**Task 4:** Testing of females carrying recombinant chromosomes for modifier phenotype. Months 6-30;  
Mice: The 800 females produced in Task 3.

**Progress:** The females from all recombinant lines produced in Task 3 have been treated with ENU, and tested for mammary tumor development. The analysis is complete for all six lines. The results are shown in Table 1 below and are published in Kohlhepp *et al.* 2000 (attached) and in press in Kohlhepp *et al.* 2001 (preprint attached). For mice from Rec1, Rec3, and Rec6 lines, neither the number or tumors (by Wilcoxon Rank sum test) nor the tumor incidence (by Fischer's exact test) was different for mice carrying the congenic recombinant chromosome and those carrying only B6 alleles in the region. For the Rec2, Rec4, and Rec5 lines, the both the number of tumors ( $p < 0.002$ , Wilcoxon Rank sum) and the number of tumor bearing mice ( $P < 0.02$ ), was different in the mice carrying the congenic recombinant chromosome as compared with those carrying only B6 alleles in the region. These results indicate that we have localized a modifier of mammary tumor susceptibility to a region of about 3 cM around the ROSA26 locus. Only the mice carrying the ROSA26 insertion show resistance to mammary tumors.

Table 1. Mammary tumor susceptibility in R26 recombinant lines.

Markers	Rec1		Rec2		Rec3		Rec4		Rec5		Rec6	
<i>Mit3</i>	129	B6	B6	B6	129	B6	B6	B6	129	B6	B6	B6
<i>Mit36</i>	129	B6	B6	B6	129	B6	B6	B6	129	B6	B6	B6
<i>Mit106</i>	129	B6	B6	B6	B6	B6	B6	B6	129	B6	B6	B6
<i>Mit105</i>	129	B6	B6	B6	B6	B6	129	B6	129	B6	B6	B6
<i>R26</i>	-	-	<i>Gtrosa26</i>	-	-	-	<i>Gtrosa26</i>	-	<i>Gtrosa26</i>	-	-	-
<i>Mit11</i>	B6	B6	129	B6	B6	B6	129	B6	129	B6	B6	B6
<i>Mit55</i>	B6	B6	129	B6	B6	B6	129	B6	B6	B6	129	B6
<i>Mit150</i>	B6	B6	129	B6	B6	B6	129	B6	B6	B6	129	B6
# of mice	12	13	9	12	16	13	14	17	5	14	15	21
% With Mammary Tumor	92	100	11	92	94	100	43	88	31	100	100	86
Ave. Mammary Tumor #	3.2	3.5	0.11*	2.8	2.1	2.8	1.1*	3.2	0.4	2.7	3.3	3.0
Ave Intestinal Tumor #	32	29	23*	32	34	32	25*	36	27	27	30	30

\*--these values are significantly different from the values obtained for the B6 sibling controls.

Because all *Min/+* mice also develop intestinal tumors, we counted intestinal tumors in these mice as well. As shown below, the mice carrying the recombinant chromosome from the Rec2 and Rec4 lines developed fewer intestinal tumors as well ( $P < 0.03$  for all comparisons, Wilcoxon Rank Sum test). However, For the Rec5 line, there was no difference in the number of intestinal tumors due to genotype on chromosome 6. To further test for an effect on intestinal tumor development, we measured intestinal tumor diameters from mice of each genotype from each of the recombinant lines. We did not measure colon tumors

because the irregular shape of those tumors makes an accurate estimate of size. The results are summarized in Table 2 below. All mice that carried the *Gtrosa26* insertion developed tumors that were smaller on average than their sibs that did not carry the insertion or the B6 *Min/+* control mice. There was no difference in tumor size observed between the B6/B6 sibs and the B6 *Min/+* control mice. The effect on tumor size was observed in all regions of the small intestine. Thus, in addition to an effect on mammary tumor susceptibility, the *Gtrosa26* insertion also has an effect on intestinal tumor growth.

Table 2: Small Intestinal Tumor Diameters of *Min/+* mice segregating for chromosome 6 intervals

Line	Chr.6	# of Mice	Ave Age	Ave Tumor Diameter (mm)			
				All regions	Duodenum	Jejunum	Ileum
Rec1	129/B6	11	101 ± 9	2.1 ± 0.9	2.3 ± 1.1	2.1 ± 0.9	1.8 ± 0.6
	B6/B6	12	103 ± 14	1.9 ± 0.9	2.0 ± 1.1	2.1 ± 0.8	1.7 ± 0.7
B6	B6/B6	18	99 ± 6	2.0 ± 0.9	2.4 ± 1.1	2.0 ± 0.8	1.7 ± 0.6
Rec2	129/B6	9	98 ± 6	1.4 ± 0.7*	1.8 ± 0.9	1.3 ± 0.5*	1.0 ± 0.3*
	B6/B6	12	99 ± 9	1.9 ± 0.8	2.0 ± 1.0	2.0 ± 0.8	1.6 ± 0.6
B6	B6/B6	20	99 ± 6	1.9 ± 0.9	2.2 ± 1.1	1.8 ± 0.8	1.6 ± 0.6
Rec4	129/B6	14	98 ± 7	1.3 ± 0.7*	1.3 ± 0.9*	1.5 ± 0.5*	1.1 ± 0.4*
	B6/B6	17	96 ± 5	1.7 ± 0.8	2.0 ± 1.0	1.6 ± 0.7	1.4 ± 0.6
B6	B6/B6	52	98 ± 4	1.8 ± 0.9	2.1 ± 1.2	1.8 ± 0.7	1.5 ± 0.5
Rec5	129/B6	16	97 ± 4	1.4 ± 0.7*	1.5 ± 0.9*	1.5 ± 0.5*	1.1 ± 0.4*
	B6/B6	14	96 ± 6	1.8 ± 0.9	2.3 ± 1.2	1.7 ± 0.7	1.5 ± 0.6
B6	B6/B6	31	99 ± 4	1.9 ± 0.9	2.2 ± 1.2	1.9 ± 0.7	1.6 ± 0.6
Rec6	129/B6	15	93 ± 6	1.8 ± 0.9	2.2 ± 1.3	1.9 ± 0.7	1.3 ± 0.4
	B6/B6	21	94 ± 7	1.8 ± 0.9	2.1 ± 1.2	1.9 ± 0.7	1.4 ± 0.5
B6	B6/B6	19	97 ± 4	1.7 ± 0.8	2.0 ± 1.1	1.8 ± 0.6	1.4 ± 0.6

\*-P<0.001 compared with B6/B6 sibs and B6*Min/+* controls.

Because all of these mice had been treated with ENU to induce mammary tumors, we wanted to test for an effect on intestinal tumor growth in mice that had not been exposed to ENU. To do this we collected intestines from male B6 *Min/+* segregating for the Rec2 interval. Tumors from all regions of the small intestine were counted and measured. The mice that carried the Rec2 chromosome developed the same number (data not shown) of intestinal tumors, but again the tumors were approximately ¾ the size of the tumors from their sibs that did not carry the insertion (Table 3). Thus, the effect of the *Gtrosa26* insertion on intestinal tumor growth is not dependent on ENU treatment.

Table 3: Intestinal Tumor Diameter in Rec2 *Min/+* mice

Genotype	# of mice	Age	Average Tumor Diameter (mm)			
			All regions	Duodenum	Jejunum	Ileum
Rec2/+	6	100 ± 1	1.5 ± 0.6*	1.8 ± 0.6	1.5 ± 0.5*	1.2 ± 0.4*
+/+	13	99 ± 2	1.9 ± 0.7	2.0 ± 1.0	1.9 ± 0.5	1.9 ± 0.5

P<0.002 compared with B6/B6 sibs.



**Task 5:** Establishment of lines carrying recombinant chromosomes to allow further testing. Each recombinant chromosome will be replicated and maintained by intercrossing mice heterozygous for the recombinant chromosome and selecting for mice homozygous, thus preserving the recombinant region intact. Months 4-36; Mice: About 4 cages/per line, or about 48 mice /line/year.

**Progress:** We have established mice that are homozygous for *Rec1* and *Rcc6*. The *Rec3* line has been discontinued. We have had difficulty in establishing a homozygous line of mice carrying the original ROSA26 congenic interval. Mice homozygous for the ROSA26 insertion (from any congenic line) are obtained at much less than the expected 25% and many homozygotes are runted and fail to breed. Therefore, we are maintaining the lines carrying the insertion in the heterozygous state.

Because the observation of reduced survival and fertility may provide a clue as to the function of the identified modifier, we have explored whether heterozygosity for the *Rec2* congenic interval had an effect on the growth of mice. We have found that, at weaning, only 40%, rather than the expected 50%, of mice from a cross of a wildtype female with a *Rec2/+* male carry the *Rec2* chromosome. Additionally, 6% (19 of 309 live births) of mice born in the *Rec2* colony are runted (defined as <50% the weight age matched sibs). All of the runts carried the *Gtrosa26* insertion. Four runts were female and 15 were male. This is a significantly higher incidence of runting than that seen in our B6 colony, where the rate of runting is less than 1%. Analysis of the growth of mice has demonstrated that the growth rates of the mice carrying the insertion is slower than that of their wild-type sibs, at least between 7 and 35 days of age. The *Rec2/+* females were 2% smaller than their sibs ( $p=0.004$ ), *Rec2/+* males were 5% smaller than their sibs ( $P=0.0001$ ). While this effect on growth is small, it is highly statistically significant, and does suggest that, in addition to runting, the *Gtrosa26* insertion has an effect on overall growth of young mice.

To determine whether the *Gtrosa26* insertion had a specific effect on the mammary gland, we measured mammary glands in female and male mice (Table 4 and 5). We chose to look at mice between 20 and 50 days of age for two reasons. First, we treat the mice with ENU when between 35-45 days of age, thus we wanted to assess mammary gland size in female mice of those ages. Second, the rapid growth and development of the mammary glands in female mice begins after 20 days of age and proceeds until about 50 day of age in B6 mice. 20 day old female *Rec2/+* mice have mammary glands that are 35% smaller than those of their age-matched B6 sibs. However, there was no difference in size in the mammary glands of *Rec2/+* female mice and wild-type female mice at 35 and 50 days of age. Thus, the differential susceptibility to ENU-induced mammary tumor development is not the result of a difference in the size of the mammary glands at the time of ENU treatment.

Table 4: Abdominal Mammary Gland Measurements of *Rec2/+* female mice

Genotype	Number of mice	Age	Average gland size (mm <sup>2</sup> )
<i>Rec2/+</i>	15	20	5.0 ± 2.1*
+/+	15		7.7 ± 2.7
<i>Rec2/+</i>	15	35	129.3 ± 33
+/+	14		118.2 ± 43
<i>Rec2/+</i>	15	50	283.7 ± 33
+/+	15		301.8 ± 49

\*  $P=0.007$  compared with +/+ age matched sibs.

In male mice, the mammary glands of the *Gtrosa26*/+ mice were significantly smaller at all ages tested. In addition, the number of mice lacking any glandular tissue in the fat pads was higher at all ages for the *Rec2*/+ mice. Thus, in the males, the effect of the *Gtrosa26* insertion on mammary gland development exerts its effect during the embryonic development of the gland, which occurs between 12 and 14 days of gestation.

**Table 6: Abdominal Mammary Gland Measurements of Male *Rec2*/+ mice.**

Genotype	Number of mice	Age	Average gland size (mm <sup>2</sup> )
<i>Rec2</i> /+	20	20	0.6 ± 0.8*
+/+	14		1.6 ± 1.2
<i>Rec2</i> /+	15	35	0.3 ± 0.6*
+/+	15		1.7 ± 1.3
<i>Rec2</i> /+	14	50	0.2 ± 0.4*
+/+	15		1.1 ± 1.2

\* P<0.01 compared with +/+ age matched sibs.

**Task 6:** Identification and testing of candidate loci by identification of ESTs in the region and mapping using recombinant chromosomes. Months 10-36, no mice.

**Progress:** As the effect on mammary tumor development mapped to within 4 cM of the *Gtrosa26* insertion, we have tested for linked modifier genes in congenic mice carrying 129-derived DNA in the region, but not the *Gtrosa26* insertion (see attached publication Kohlhepp *et al.* in press). These experiments provide evidence that the effect seen in the *Gtrosa26* mice is not the result of linked modifier genes, but is the result of the insertion. In brief, mice congenic for 129-derived DNA in the *Gtrosa26* interval were as susceptible to mammary tumor development as were their siblings not carrying 129 DNA in the congenic interval. Thus, we found no evidence for a linked modifier locus. The leads to the conclusion that it is the insertion itself that results in the effects on mammary tumor development, intestinal tumor growth, growth rate of the mice, and mammary gland size and development. This effect could be the result of the expression of the  $\beta$ -galactosidase-neomycin phosphotransferase protein, the decreased expression of the two non-coding transcripts encoded by the *Gtrosa26* locus or disrupted expression of nearby unidentified loci.

**Task 7.** Production and testing of mice transgenic for candidate modifier genes. This Task will be dependent on the progress made in Tasks 1-5. If the number of candidates gets down to 1-2, they will be tested for function as transgenes.

**Progress:** We have obtained and are testing mice carrying the *Gtrosa26* insertion that have been modified such that the mRNA encoding the  $\beta$ -gal-neomycin phosphotransferase protein is not expressed. We are currently testing (under supplemental funding) whether these mice are susceptible or resistant to tumor mammary tumor development. If they are as susceptible as are B6 mice, then we would conclude that resistance to tumor development is the result of the function of the fusion protein. If the mice are resistant, then we would conclude that resistance does not require the fusion protein, but is the result of one of the other effects of the insertion.

## **Aim 2.**

**Task 1:** Testing tumors for allele loss at *Apc*. Months 1-6, no mice, this will utilize archived tumor samples.

**Progress:** We have begun testing tumors for LOH at *Apc*. The UW Comprehensive Cancer Center and the Environmental Health Sciences Center have jointly established a Laser Capture Microscopy Facility. We have collected samples from some tumors for analysis. Careful histological analysis of the mammary tumors indicated that without a laser capture device it would be difficult to obtain samples that were mainly tumor tissue and did not include large amounts of normal tissue. By using Laser Capture, we can obtain smaller samples from numerous sites within the tumor and decrease the amount of normal tissue contamination. We have determined the amount of captured areas that are needed for reliable analysis and are beginning the LOH studies. In order to insure that the assay is quantitative, careful titration of the amount of input DNA is required prior to testing for allele loss. One difficulty that we have detected is the large amount of infiltration of the tumor tissue with inflammatory cells. We are currently analyzing the results of the experiments we have done. We are in the process of requesting further funding to support these studies.

**Task 2:** Testing tumors for allele loss in modifier region. Months 1-36, no mice. As the modifier interval is narrowing by analysis of the modifier region, we can test more directly for loss of the modifier locus.

**Progress:** As our experiments indicate that it is the presence of the *Gtrosa26* insertion that is responsible for tumor resistance, we have tested for loss of the *Gtrosa26* insertion by testing for  $\beta$ -galactosidase activity in mammary tumors. The rationale for these experiments is that if the insertion sequences have been lost, the tumor cells would no longer express  $\beta$ -galactosidase. In all cases tested (currently over 10 tumors), the tumor cells express high levels of bacterial  $\beta$ -galactosidase. Thus, loss of the insertion, or loss of function of the insertion sequences is not required for tumor development.

**Task 3.** Compilation of results and preparation of publication. Months 6-8, 34-36.

**Progress:** Two publications have been published and two more are in preparation. The first publication describes the mapping of the effect on chromosome 6 to within 4 cM of the *Gtrosa26* insertion (Kohlhepp *et al.* Mammalian Genome, 11:1058-1062, 2000). The second publication provides evidence that the resistant to mammary tumor development is the result of the *Gtrosa26* insertion (Kohlhepp *et al.* Mammalian Genome, in press 2001). The two manuscripts in preparation report the effect of the *Gtrosa26* insertion on intestinal tumor development and the effect on growth of the mice and mammary gland growth. These papers will be submitted over the summer.

## KEY RESEARCH ACCOMPLISHMENTS

- We have identified 12 markers on chromosome 6 that are polymorphic between B6 and 129. We have determined the map location of these markers. In some cases, the map position differs from the published order.
- We have generated and characterized eight congenic lines carrying a region of 129-derived DNA on the B6 background.
- We have determined that the effect on mammary tumor susceptibility is the result of the *Gtrosa26* insertion, and not to a linked modifier locus.
- We have determined that the ROSA26, Rec2, Rec4, and Rec5 mice also carry a modifier of intestinal tumor development.
- We have determined that the ROSA26-associated modifier also affects the growth of the mammary glands and body weight, in male mice and prepubertal female mice.

## REPORTABLE OUTCOMES

### Oral Presentation:

"Genetic Analysis of Mammary Tumor Susceptibility in *Min*/<sup>+</sup> Mice", Kohlhepp, R., Hegge, L., and Moser, A.R., at "Genetics, Genomics and Molecules, Madison, WI, 5/23-25/99.

"ROSA26/<sup>+</sup> *Min*/<sup>+</sup> mice are resistant to mammary tumor development" At Era of Hope DOD Breast Cancer Research Program Meeting, Atlanta, GA June 8-11/2000.

"The effect of modifier loci on mammary and intestinal tumor development in *Apc*<sup>*Min*</sup>/<sup>+</sup> mice" Department of Biological Sciences, University of South Carolina, Columbia, SC. 7/18/2000.

"The ROSA26 Insertion Results in Resistance to Mammary Tumor Development in *Apc*<sup>*Min*</sup>/<sup>+</sup> Mice" the Molecular and Cellular Biology Graduate Program, University of Massachusetts–Amherst, MA.

"The effect of the ROSA26 insertion on mammary tumorigenesis in *Apc*<sup>*Min*</sup>/<sup>+</sup> mice". At the monthly meeting of the NIH Mouse Models of Mammary Cancer Collective, Bethesda, MD 11/2000.

"Mammary Tumor Development in *Apc*<sup>*Min*</sup>/<sup>+</sup> mice" At Lombardi Cancer Center, Georgetown University, Washington, DC, 11/2000.

"Investigation of a Novel Genetic Modifier of Mammary Tumor Susceptibility Associated with the ROSA26 Insertion" At University of Connecticut Health Center, School of Medicine, 2/2001.

### Poster Presentations:

"Mapping of a Locus Affecting Susceptibility to Mammary Tumor Development", Kohlhepp, R., Nett, J., Hegge, L., and Moser, A.R., at "Modeling Human Mammary Cancer in Mice" The Jackson Laboratory, Bar Harbor, ME, 10/5-8/99.

"ROSA 26 Mice are resistant to mammary tumor formation" R. L. Kohlhepp, L. F. Hegge, J. E. Nett, and A. R. Moser, at The AACR Annual Meeting, San Francisco, CA, 4/1-5/2000.

"ROSA26/<sup>+</sup> *Min*/<sup>+</sup> mice are resistant to mammary tumor development" At Era of Hope DOD Breast Cancer Research Program Meeting, Atlanta, GA June 8-11/2000.

### Manuscripts:

Kohlhepp, RL, Hegge, LF, Nett, JE, and Moser, AR. ROSA26/<sup>+</sup> mice are resistant to *Min*-induced mammary and intestinal tumor development. *Mammalian Genome*, 11:1058-1062, 2000

Kohlhepp, RL, Hegge, LF, and Moser, AR The ROSA26 *LacZ-neoR* insertion confers resistance to mammary tumors in *Apc*<sup>*Min*</sup>/<sup>+</sup> mice. In press, *Mammalian Genome*.

We have established a congenic line of mice that carry the ROSA26 insertion with a minimal interval of 129-derived DNA.

## CONCLUSIONS

We have identified 6 mice carrying recombinant chromosomes from the congenic region carried by the B6.ROSA26 mice. We have established lines from each of these mice and have fully tested each of the chromosomes for the effect on mammary tumor development in *Min*/<sup>+</sup> mice. In addition, we have produced and tested two lines of mice carrying 129 DNA in the *Gtrosa26* region, but not the insertion, on the B6

background for mammary tumor susceptibility. The results of these studies indicate that the resistance to tumor development in the *Gtrosa26*<sup>+</sup>/<sub>+</sub> mice is due to the presence of the insertion, and not to a linked modifier locus. Under this funding, we have begun and will continue (under separate funding) experiments designed to determine whether the expression of the fusion protein encoded by the *Gtrosa26* insertion is required for mammary tumor resistance.

We have also determined the insertion affects the growth of intestinal tumors, in both ENU-treated and untreated mice. On average the intestinal tumors in *Gtrosa26*<sup>+</sup>/<sub>+</sub> mice are 75% the diameter of tumors from wild-type mice. Further studies have shown that the *Gtrosa26* insertion also affects the development of the mammary gland in prepubertal female mice and in male mice. Mice carrying the insertion have smaller mammary glands than do the age and sex matched controls. *Gtrosa26*<sup>+</sup>/<sub>+</sub> mice are also significantly smaller than their wild-type sibs at all ages tested. In addition, we found an increased incidence of runts in the *Gtrosa26* colony as compared to the B6 colony. All of the runts were shown to carry the *Gtrosa26* insertion. Thus, the insertion has an effect on development of mammary tumors, and growth of the mice, the mammary glands and intestinal tumors.

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- Kohlhepp, RL, Hegge, LF, and Moser, AR. The ROSA26 *LacZ-neoR* insertion confers resistance to mammary tumors in *Apc*<sup>*Min*<sup>+</sup></sup> mice. In press, *Mammalian Genome*.

## APPENDICES

- Reprint of: Kohlhepp, RL, Hegge, LF, Nett, JE, and Moser, AR. ROSA26<sup>+</sup>/<sub>+</sub> mice are resistant to *Min*-induced mammary and intestinal tumor development. *Mammalian Genome*, 11:1058-1062, 2000.
- Preprint of: Kohlhepp, RL, Hegge, LF, and Moser, AR. The ROSA26 *LacZ-neoR* insertion confers resistance to mammary tumors in *Apc*<sup>*Min*<sup>+</sup></sup> mice. In press, *Mammalian Genome*.

## ROSA26 mice carry a modifier of *Min*-induced mammary and intestinal tumor development

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**Abstract.** B6.129S7-*Gtrosa26* (B6.R26) mice carry a *LacZ-neoR* insertion on Chromosome (Chr) 6, made by promoter trapping with 129 ES cells. Female C57BL/6J *Apc*<sup>*Min*</sup>/+ (B6*Min*/+) mice are highly susceptible to intestinal tumors and to the induction of mammary tumors after treatment with ethylnitrosourea (ENU). However, B6.R26/+ *Min*/+ females develop fewer mammary and intestinal tumors after ENU treatment than do B6 *Min*/+ mice. B6.R26/+ mice from two independently derived congenic lines show this modifier effect. Each of these congenic lines carries approximately 20 cM of 129-derived DNA flanking the insertion, raising the possibility that the resistance is due to a linked modifier locus. To further map the modifier locus, we have generated several lines of mice carrying different regions of the congenic interval. We have found that resistance to mammary and intestinal tumors in ENU-treated *Min*/+ mice maps to a minimum 4-cM interval that includes the ROSA26 *LacZ-neoR* insertion. Therefore, the resistance to tumor development is due to either the ROSA26 insertion or a very tightly linked modifier locus.

### Introduction

B6 *Min*/+ mice carry a dominantly inherited germline mutation at *Apc* and are predisposed to spontaneous intestinal and mammary tumors (Moser et al. 1990, 1993; Su et al. 1992). B6 *Min*/+ mice develop multiple intestinal tumors and rarely survive beyond 120 days. Even given this short life span, 5% of female *Min*/+ mice on a B6 background develop spontaneous mammary tumors. When treated with ENU, a direct-acting alkylating agent, about 90% of female B6 *Min*/+ mice develop mammary tumors, with an average of three mammary tumors per mouse. This is clearly a *Min*-specific effect, as wild-type mice do not develop mammary tumors under this protocol. Genetic background has a strong effect on the susceptibility to intestinal tumors in *Min*/+ mice (Moser et al. 1995). Thus, *Min*/+ mice are a useful model with which to identify factors, either genetic or environmental, that affect mammary and intestinal tumor development.

B6.129S7-*Gtrosa26* (ROSA26) mice carry a *LacZ-neoR* insertion on Chr 6 (Gould and Dove 1997), made by random retroviral insertion and exon trapping with 129 ES cells (Friedrich and Soriano 1991). ROSA26 mice express  $\beta$ -galactosidase ( $\beta$ -gal) ubiquitously, making them a useful tool for chimera and transplant studies (Abrahamson et al. 1998; Borthwick et al. 1999; Gould and Dove 1996, 1997; Matsusaka et al. 1999; Wong et al. 1996; Zambrowicz et al. 1997). ROSA26 mice on a mixed 129, B6 background are reported to have no apparent phenotypic abnormalities (Zambrowicz et al. 1997). Congenic B6.129S7-*Gtrosa26* (B6.R26/+) mice have been generated by backcrossing to C57BL/6J (B6) mice (Gould and Dove 1996) to allow use as a marker strain in chimeras.

Prior to using  $\beta$ -galactosidase as a cellular marker in the mammary tumors of *Min*/+ mice, we tested B6.R26/+ *Min*/+ mice for mammary tumor susceptibility. We have found that B6.R26/+ mice are very resistant to *Min*-induced mammary and intestinal tumor formation. Two independently derived lines of B6.R26/+ mice retain 20 cM of 129-derived DNA flanking the *LacZ-neoR* insertion site. To begin to localize the modifier conferring resistance in these B6.R26/+ mice, we have generated four lines that carry different recombinant congenic intervals from the ROSA26 congenic region. We have found that resistance maps to a 4-cM interval that contains the *LacZ-neoR* insertion. Thus, the resistance to tumor formation is due either to the ROSA26 insertion or to a very tightly linked modifier locus.

### Materials and methods

**Mice.** All mice were bred at the University of Wisconsin Medical School Animal Care Facility. The *Min* pedigree is maintained by backcrossing *Min*/+ males to B6 females. B6 *Min*/+ parents for these experiments were from generations N36–N46. Animals were genotyped for *Min* by PCR by using an allele-specific PCR assay (Dietrich et al. 1993). The two B6.129S7-*Gtrosa26* lines (R26-1 and R26-2) were derived independently from the same 129.B6-*Gtrosa26*/+ founder. For the first 16 backcross generations, the R26/+ mice were identified by screening for mice expressing  $\beta$ -gal in the blood or tissue (Gould and Dove 1997). After that time, R26/+ mice have been identified by a PCR assay for the presence of the R26 insertion (see below). Both lines are maintained by crossing B6 females with B6 R26/+ males. The ROSA26 mice used for the crosses to the B6.*Min*/+ mice were from the N12–N14 generations.

Recombinant congenic lines Rec1, Rec2, and Rec3 are derived from the R26-1 colony. The Rec6 recombinant chromosome was identified within the Rec2 colony. Each congenic recombinant line is maintained by crossing mice heterozygous for the congenic interval with B6 mice. F<sub>1</sub> mice were produced by crossing heterozygous females from R26-1, Rec1, Rec2, Rec3, or Rec6 to B6 *Min*/+ males. For the R26-2 experiments, mice were produced by crossing R26/+ females with B6.*Min*/+ males or by crossing B6 females with R26/+ *Min*/+ males.

**PCR genotyping.** The presence of the ROSA26 insertion was confirmed by testing for the presence of *LacZ* by using primers *LacZ* 3F (5'-CAGAGCGGGTAACTGGCTCGGATTAG-3') and *LacZ* 2R (5'-GACACCAGACCAACTGGTAATGGTAGC-3'). PCR was run on an MJ Research PTC-100 Programmable thermal controller. The PCR conditions were: 1.25 mM MgCl<sub>2</sub>, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.2 mM dNTPs, 0.8  $\mu$ M of each primer, 1.25 units of Promega Taq polymerase in storage buffer A, and genomic DNA for a total reaction volume of 25  $\mu$ l. Initial denaturation at 92°C for 2 min, followed by 30 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 45 s, and extension at 72°C for 45 s, followed by extension at 72°C for 5 min. PCR products were electrophoresed in 2% agarose and visualized with EtBr staining. The extent of the 129-derived congenic interval was determined with SSLP markers on Chr 6 (Dietrich et al. 1992).

**Table 1.** The incidence and number of mammary and intestinal tumors for *Min*/+ mice from the R26-1 and R26-2 congenic lines segregating for R26.

Mouse line	Genotype	Number of mice	Number with mammary tumors (%)	Mammary tumors (mean $\pm$ s.d.)	Intestinal tumors (mean $\pm$ s.d.)
R26-1	R26/+	17	7 (41) <sup>a</sup>	0.5 $\pm$ 0.6 <sup>b</sup>	32 $\pm$ 14
	+/+	22	16 (73)	1.8 $\pm$ 1.5	36 $\pm$ 12
B6		32	29 (91)	2.8 $\pm$ 1.9	33 $\pm$ 10
	R26/+	18	8 (45) <sup>c</sup>	0.8 $\pm$ 1.0 <sup>d</sup>	28 $\pm$ 6 <sup>e</sup>
	+/+	18	15 (83)	2.2 $\pm$ 1.6	39 $\pm$ 11
B6		81	71 (88)	2.6 $\pm$ 1.6	33 $\pm$ 8

<sup>a</sup>  $P = 4 \times 10^{-4}$  compared with B6 *Min*/+ controls.<sup>b</sup>  $P = 0.004$  compared with +/+ sibs and  $P = 8 \times 10^{-6}$  compared with B6 *Min*/+ controls.<sup>c</sup>  $P = 0.007$  compared with +/+ sibs and  $P = 4 \times 10^{-5}$  compared with B6 *Min*/+ controls.<sup>d</sup>  $P = 0.03$  compared with +/+ sibs and  $P = 0.002$  compared with B6 *Min*/+ controls.<sup>e</sup>  $P = 0.002$  compared with +/+ sibs and  $P = 0.006$  compared with B6 *Min*/+ controls.

All mice had intestinal tumors. The B6 mice are B6 *Min*/+ mice that were treated with ENU at the same time as the mice from each R26 line. All mice designated as R26/+ were heterozygous 129/B6 at markers spanning the *D6Mit3*–*D6Mit55* interval. Those designated +/+ carried only B6 alleles at *D6Mit3*–*D6Mit55*. Tumor multiplicities were compared using Wilcoxon rank sum test. Tumor incidences were compared using Fisher's exact test. All  $P$  values are two sided.

jection of 50 mg/kg body weight ENU (Sigma Chemical, USA) between 35 and 45 days of age (Moser et al. 1993). Mice were palpated weekly to detect mammary tumors. B6 *Min*/+ mice from the *Min* colony were included in all rounds of mutagenesis as a control for the effects of ENU. Mice were sacrificed when moribund or 60 days after ENU treatment. The exception was mice in the first study, which were sacrificed when moribund. Mammary tumors were counted and collected at the time of sacrifice. Tumors were fixed in formalin for sectioning and histological analysis. Intestines were also collected at the time of necropsy and processed for tumor counts (Moser et al. 1990). The tumors in 4-cm sections from the duodenum, jejunum, and ileum and the entire colon were counted. All animals were scored without knowledge of genotype.

**Statistical analysis.** Analyses were performed with the MSTAT computer program, provided by Norman Drinkwater at the McArdle Laboratory for Cancer Research. For tests of tumor multiplicity, the Wilcoxon rank sum test was used. Fisher's exact test was used to compare tumor incidence.

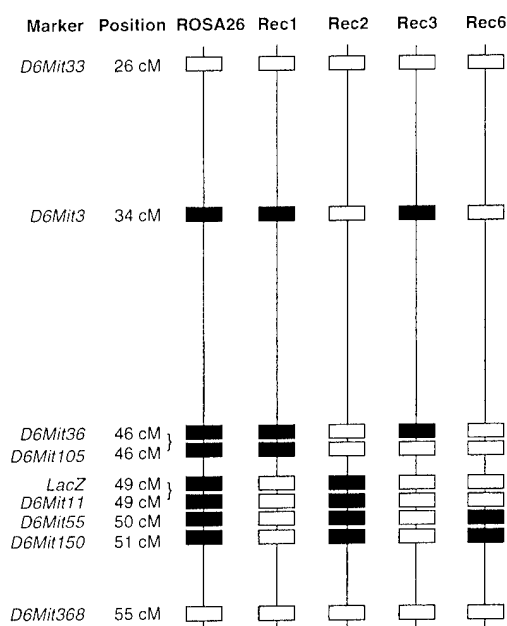
## Results

**ROSA26 *Min*/+ mice are resistant to ENU-induced mammary tumor formation.** Two B6 congenic lines heterozygous for the ROSA26 insertion, R26-1 and R26-2, were maintained in the lab for mammary transplant experiments. To test for development of mammary tumors, we generated R26/+ *Min*/+ female mice from the R26-1 line and treated them with ENU (Table 1). The mice carrying the ROSA26 insertion (R26/+ *Min*/+) developed significantly fewer mammary tumors than did their sibs not carrying the insertion (+/+ *Min*/+) or than did the B6 *Min*/+ control animals. The number of mice with tumors was also less for the R26/+ *Min*/+ mice than for the B6 controls. The +/+ *Min*/+ mice developed mammary tumors at approximately the same incidence ( $P = 0.14$ ) and multiplicity ( $P = 0.06$ ) as did B6 *Min*/+ control mice. This decreased susceptibility to mammary tumors was seen even though the R26/+ *Min*/+ mice survived for an average of 86 days after ENU as compared with 71 days for the +/+ *Min*/+ mice ( $P = 0.0007$ ) or 66 days for the B6 *Min*/+ control mice ( $P < 9 \times 10^{-6}$ ). Some of the mammary tumors were tested for expression of  $\beta$ -galactosidase by staining with X-gal, and in all cases, the tumors were positive for enzyme activity (data not shown). Thus, tumor development does not require loss of expression of  $\beta$ -geo.

Because R26/+ mice from the R26-1 line were surprisingly resistant to mammary tumors, we tested the mice from the R26-2 line (Table 1). This line was derived from the same 129, B6 ROSA26/+ founder, but had been independently backcrossed for at least 13 generations at the time of the experiments. We generated R26/+ *Min*/+ females from the R26-2 line and treated them with ENU. In confirmation of our results with the R26-1 line, both the incidence and multiplicity of mammary tumors were significantly reduced in the R26/+ *Min*/+ mice relative to their +/+ *Min*/+ sibs or the B6 *Min*/+ mice (Table 1). Again, the +/+ *Min*/+ mice were not different from the B6 *Min*/+ control mice in either tumor incidence ( $P = 1$ ) or multiplicity ( $P = 0.41$ ).

**ENU-treated ROSA26 *Min*/+ mice are resistant to intestinal tumor formation.** ENU-treated R26/+ *Min*/+ mice from the R26-2 line also developed significantly fewer intestinal tumors (Table 1) than did the +/+ *Min*/+ sibs or the B6 *Min*/+ controls (Table 1). The number of intestinal tumors in the +/+ *Min*/+ mice from the R26-2 line was not different from the B6 *Min*/+ controls ( $P = 0.06$ ). In contrast, the R26/+ *Min*/+ mice from the R26-1 line developed the same number of intestinal tumors as did the B6 *Min*/+ control mice. However, in the R26-1 experiments, the mice were killed when moribund rather than at a set time after ENU. The R26/+ *Min*/+ mice from the R26-1 line survived for an average of 15 days longer after ENU treatment as compared with the +/+ *Min*/+ sibs or 20 days longer compared with the B6 *Min*/+ control mice. In contrast, the R26/+ *Min*/+ mice from the R26-2 line were sacrificed at approximately the same time as the +/+ *Min*/+ mice from that cross. The increased lifespan of the R26/+ mice from the R26-1 line may have allowed for the growth of more intestinal tumors.

**Fine mapping of the ROSA26-associated modifier.** The tumor resistance of the R26/+ *Min*/+ mice from both lines raised the possibility that a modifier of tumor susceptibility had been created by the insertion event or that a modifier allele from 129 was linked to the insertion. To determine the extent of 129 DNA flanking the *LacZ-neoR* insertion, we genotyped R26/+ mice from both lines at SSLP markers along Chr 6. We found that both lines retained a similar, approximately 20-cM, segment of 129 DNA spanning from *D6Mit3* to *D6Mit150* (Fig. 1). To narrow the modifier interval, we identified mice from the R26-1 line recombinant within the ROSA26 congenic region. We produced four lines, each carrying a different recombinant segment of the ROSA26 congenic interval (Fig. 1). Only the Rec2 mice carry the *LacZ-neoR* insertion and express  $\beta$ -galactosidase. Female mice from each of these lines heterozygous for the 129-derived interval were crossed with B6 *Min*/+ males, and the female *Min*/+ progeny were treated with ENU to assess mammary and intestinal tumor susceptibility (Table 2). All of the mice were sacrificed at approximately the same time after ENU treatment, and the number of mammary and intestinal tumors was counted. Only the Rec2/+ *Min*/+ mice developed significantly fewer mammary and intestinal tumors than did their B6/B6 *Min*/+ siblings. Although only one of nine Rec2/+ *Min*/+ mice developed mammary tumors, the incidence and multiplicity are not significantly different in R26/+ *Min*/+ mice from their parental R26-1 line ( $P = 0.19$  and  $P = 0.12$  respectively). The Rec1, Rec3, and Rec6 congenic intervals had no effect on either mammary or intestinal tumor number. Because mice carrying the Rec1 congenic interval were as susceptible to mammary and intestinal tumors as the B6 *Min*/+ control animals, the modifier must map distal to *D6Mit105*. Similarly, because mice carrying the Rec6 congenic interval were as susceptible to mammary and intestinal tumors as B6 *Min*/+ controls, the modifier must map proximal to *D6Mit55*. This limits the modifier to the region between *D6Mit105* and *D6Mit55*, which spans about 4 cM and includes the *LacZ-neoR* insertion.



**Fig. 1.** A representation of a portion of Chr 6 is shown for the ROSA26 mice and the mice from each of the Recombinant lines. The position from the centromere for each marker was obtained from Mouse Genome Database (MGD), Mouse Genome Informatics, The Jackson Laboratory, Bar Harbor, Maine. World Wide Web (URL: <http://www.informatics.jax.org/>) (3/01/2000). The order of the markers is the same as that obtained from MGD except for *D6Mit36* and *D6Mit105*. As the Rec3 mice carry the 129 allele of *D6Mit3* and *D6Mit36* and the B6 allele of *D6Mit105*, the most likely order is as shown. A filled box indicates a 129 allele at the locus; an open box indicates a B6 allele. For *LacZ*, a filled box indicates the presence of the insertion; an open box the absence of the insertion.

**Table 2.** The incidence and the multiplicity of mammary and intestinal tumors for ENU-treated *Min/+* female mice segregating for the recombinant Chr 6.

Line	Genotype	Number of mice	Number with mammary tumors (%)	Mammary tumors (mean $\pm$ s.d.)	Intestinal tumors (mean $\pm$ s.d.)
Rec1	129/B6	11	10 (91)	3.2 $\pm$ 1.9	32 $\pm$ 7
	B6/B6	12	12 (100)	3.7 $\pm$ 1.7	29 $\pm$ 7
Rec2	129/B6	9	1 (11) <sup>a</sup>	0.1 $\pm$ 0.3 <sup>b</sup>	23 $\pm$ 13 <sup>c</sup>
	B6/B6	12	11 (92)	2.8 $\pm$ 1.9	32 $\pm$ 6
Rec3	129/B6	16	15 (94)	2.1 $\pm$ 1.4	34 $\pm$ 10
	B6/B6	13	13 (100)	2.8 $\pm$ 1.4	32 $\pm$ 7
Rec6	129/B6	15	15 (100)	3.3 $\pm$ 1.6	30 $\pm$ 6
	B6/B6	21	18 (86)	3.0 $\pm$ 2.1	30 $\pm$ 7
B6	B6/B6	99	89 (90)	3.1 $\pm$ 1.9	32 $\pm$ 9

<sup>a</sup>  $P = 4 \times 10^{-4}$  compared with B6/B6 sibs.

<sup>b</sup>  $P = 0.0003$  compared with B6/B6 sibs.

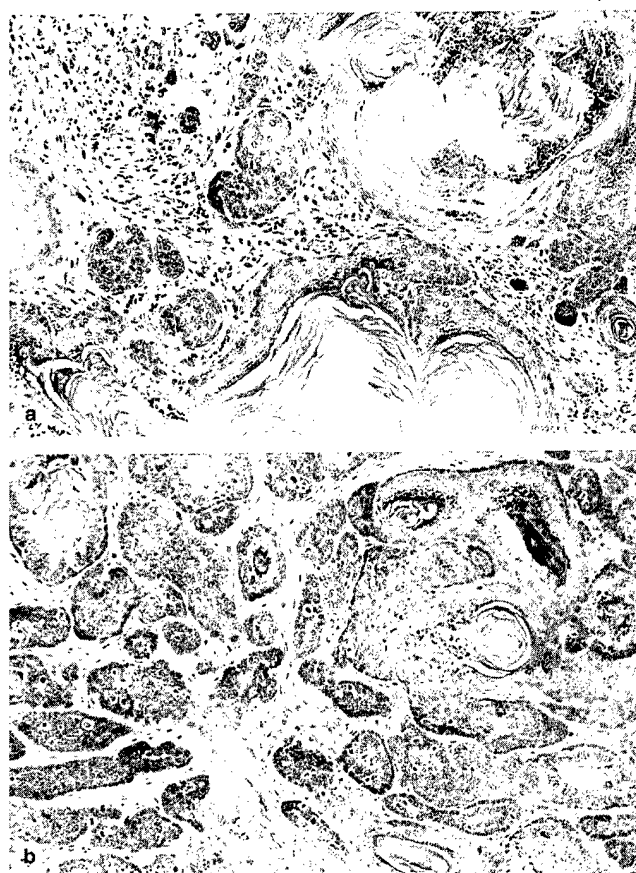
<sup>c</sup>  $P = 0.03$  compared with B6/B6 sibs.

All mice had intestinal tumors. The mice that inherited the congenic chromosome are designated as 129/B6, while those that inherited the B6 chromosome are designated B6/B6. The B6 mice are B6 *Min/+* female mice treated with ENU at the same time as the congenic mice. Tumor multiplicities were compared using Wilcoxon rank sum test. Tumor incidences were compared using Fisher's exact test. All  $P$  values are two sided.

**Mammary tumor histology.** While the ROSA26 modifier had a significant effect on mammary tumor susceptibility, the tumors that did develop were indistinguishable from those of the B6 *Min/+* mice. Mammary tumors from mice R26/+ and +/+ from all lines were classified as squamous cell carcinomas (Fig. 2).

## Discussion

These results indicate that R26/+ mice from two B6.R26 congenic lines and the B6.Rec2 recombinant line carry a dominantly acting



**Fig. 2.** Representative H&E stained histological sections of mammary tumors from B6 *Min/+* (a) and from a Rec2/+ *Min/+* mouse (b) are shown. Both tumors are squamous cell carcinomas.

modifier allele that confers resistance to mammary and intestinal tumors in *Min/+* mice. This is the first report of a modifier that affects both mammary and intestinal tumor development in *Min/+* mice. As the modifier effect on both mammary and intestinal tumor development has been noted only in ENU-treated mice, we cannot rule out that the modifier affects the response of these tissues to ENU treatment. Since only about 5% of *Min/+* females develop mammary tumors spontaneously (Moser et al. 1995), it would be difficult to assay for an effect of the ROSA26-associated modifier on spontaneous mammary tumor development. No difference in intestinal tumor number is apparent in R26/+ *Min/+* mice that are not treated with ENU (Gould and Dove 1997, data not shown). However, a small difference in intestinal tumor number in untreated mice might not have been seen in these studies.

The effect of the ROSA26-associated modifier on mammary tumor susceptibility was quite strong, resulting in more than a twofold reduction in mammary tumor number and in the number of mice developing tumors. Even the R26/+ mice from the R26-1 line, which survived for at least 2 weeks longer than their +/+ sibs, developed significantly fewer tumors, and only 40% of the R26/+ *Min/+* mice developed mammary tumors.

In contrast, the effect on intestinal tumor number was smaller and was seen only when the time after ENU treatment was similar for the ROSA26/+ and control mice. Our results suggest that the modifier may affect the growth rate of intestinal tumors. An effect on growth rate could explain why the effect on intestinal tumor number is more easily detected in ENU-treated R26/+ *Min/+* mice. ENU treatment of B6 *Min/+* mice results in an increased number of intestinal tumors (Shoemaker et al. 1997), but if the growth rate were slower in R26/+ mice, the new tumors induced by ENU



might not be apparent in the 60 days post treatment that the mice were followed. This might also explain why the intestinal tumor numbers in the R26/+ mice from the R26-1 line were not different from the +/+ controls. In the R26-1 experiment, the increased survival time of the R26/+ mice would have allowed more time for tumors to appear. The Rec2 mice, which were derived from the R26-1 line, show the effect on intestinal tumor number, suggesting that the R26-1 line does carry a modifier that would affect intestinal tumor number. An effect on both multiplicity and growth rate of intestinal tumors has been demonstrated for *Mom1*, a modifier of intestinal tumor susceptibility in *Min*/+ mice (Gould and Dove 1996). However, unlike the ROSA26 modifier, *Mom1* does not affect mammary tumor susceptibility in *Min*/+ mice (data not shown).

The modifier we have identified may be due to the insertion or to a tightly linked 129-derived modifier allele. At present, we cannot differentiate between those two hypotheses. The information available on the ROSA26 insertion site provides no obvious explanation for the effect on tumor development. The *LacZ-neoR* reporter gene is inserted into a region that produces three transcripts (Zambrowicz et al. 1997). Two of the transcripts share a promoter, have identical 5' ends, and do not contain any significant ORFs. The insertion disrupts both of these transcripts, and *LacZ-neoR* expression is driven by the endogenous shared promoter. Transcripts 1 and 2 are not expressed in homozygous ROSA26 mice. The expression of these transcripts has not been studied in ROSA26 heterozygous mice. The third transcript, transcript AS, originates from the reverse strand and potentially encodes a novel 505 amino acid protein of unknown function. Transcript AS is expressed in ROSA26 homozygous mice in multiple tissues at apparently normal levels. However, its expression in the mammary gland or intestine has not been determined. The functions of transcripts 1 and 2 are unknown, but as transcript 2 overlaps transcript AS, it may function as an antisense regulator of transcript AS. In addition, the ROSA26 insertion results in the ubiquitous expression of a fusion protein with  $\beta$ -galactosidase and neomycin phosphotransferase functions. It is not possible to rule out the expression of this fusion protein as the cause of the tumor resistance in R26/+ mice, although tumor development does not require the loss of expression of the  $\beta$ -geo.

The tumor resistance may also be due to an effect of the insertion on the expression of neighboring genes. Examples of insertions affecting the regulation of neighboring genes have been reported in other mice carrying transgenes or targeted mutations (Barrow and Capecchi 1996; Olson et al. 1996). This leaves open the possibility that an unidentified gene important in breast cancer development maps to the region and is affected by the insertion, either directly or indirectly. This region of mouse Chr 6 is homologous to human Chr 3p25, which is commonly a target of LOH in human breast cancers (Matsumoto et al. 1997). The VHL (Von Hippel-Lindau) locus maps to human 3p25 and in the mouse between *D6Mit11* and *D6Mit55*, placing it within the minimal modifier region (Street et al. 1998). However, it does not frequently have point mutations in breast cancers (Gnarra et al. 1994) and is not within the most commonly deleted region in breast cancers (Matsumoto et al. 1997).

These experiments also characterize the extent of the congenic interval (approximately 20 cM) that was retained in two independently derived lines of ROSA26 congenic mice. Our R26-1 line is derived from the same line as the B6.129S7-*Gtrosa26* mice available from The Jackson Laboratory. We tested DNA from the B6.129S7-*Gtrosa26* mice available from The Jackson Laboratory and found that they also carry the same minimum interval (*D6Mit36*-*D6Mit150*) of 129-derived DNA (data not shown). Therefore, those B6.129S7-*Gtrosa26* mice carry at least the modifier identified here, in addition to 129-derived alleles at a large number of loci.

The significant effect of the ROSA26-associated modifier on

mammary and intestinal tumor development reported here was noted mainly because tumor number can be quantitated. While the mechanism of tumor resistance is currently unknown, the ROSA26-associated modifier clearly has an effect on tumor development in at least two tissues in *Min*/+ mice. We do not know if this modifier effect is restricted to *Min*-induced tumors or to these two tissues. The wide use of these valuable mice in chimeric and transplant studies makes further characterization of the ROSA26-associated modifier pertinent.

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## The ROSA26 *LacZ-neo<sup>R</sup>* insertion confers resistance to mammary tumors in *Apc<sup>Min/+</sup>* mice

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**Abstract.** B6.129S7-*Gtosa26* (ROSA26) mice carry a *LacZ-neo<sup>R</sup>* insertion on Chromosome (Chr) 6, made by promoter trapping with AB1 129 ES cells. Female C57BL/6J *Apc<sup>Min/+</sup>* (B6 *Min/+*) mice are very susceptible to the induction of mammary tumors after treatment with ethylnitrosourea (ENU). However, ENU-treated B6 mice carrying both *Apc<sup>Min</sup>* and ROSA26 are resistant to mammary tumor formation. Thus, ROSA26 mice carry a modifier of *Min*-induced mammary tumor susceptibility. We have previously mapped the modifier to a 4-cM interval of 129-derived DNA that also contains the ROSA26 insertion. Here we report additional evidence for the effect of the ROSA26 insertion on mammary tumor formation. To test the hypothesis that the resistance was due to a linked modifier locus, we utilized two approaches. We have derived and tested two lines of mice that are congenic for 129-derived DNA within the minimal modifier interval and show that they are as susceptible to mammary tumors as are B6 mice. Additionally, we analyzed a backcross population segregating for the insertion and show that mice carrying the insertion are more resistant to mammary tumor development than are mice not carrying the insertion. Thus, the resistance is not due to a 129-derived modifier allele, but must be due to the ROSA26 insertion. In addition, the effect of the ROSA26 insertion can be detected in a backcross population segregating for other mammary modifiers.

### Introduction

*Min/+* (multiple intestinal neoplasia) mice carry a mutation in the *Apc* (adenomatous polyposis coli) gene and are predisposed to develop intestinal and mammary tumors (Moser et al. 1990, 1993; Su et al. 1992). On a C57BL/6J (B6) background, all *Apc<sup>Min/+</sup>* (*Min/+*) mice develop intestinal tumors, and about 5% of *Min/+* female mice develop mammary tumors by 100 days of age. When treated with ENU, a direct alkylating agent, approximately 90% of B6 *Min/+* female mice develop mammary tumors within 65 days of treatment with an average of three tumors per mouse (Moser et al. 1993). Thus, *Min/+* mice are a good model system for the identification of genes that modify risk of mammary tumor development.

We have previously reported that when B6 *Min/+* mice are crossed to B6.129 S7-*Gtosa26* (ROSA26) mice, the number of mammary tumors is significantly reduced in the ROSA26/+ *Min/+* mice as compared with their +/+ *Min/+* sibs (Kohlhepp et al. 2000). Thus, B6.ROSA26/+ mice carry a modifier of *Min*-induced mammary tumor development. ROSA26 mice carry a *LacZ-neo<sup>R</sup>* insertion on Chromosome (Chr) 6 (Gould and Dove 1997), made by random retroviral insertion and exon trapping using 129 ES cells (Friedrich and Soriano 1991). ROSA26 mice express  $\beta$ -ga-

lactosidase ( $\beta$ -gal) ubiquitously, making them a useful tool for chimera and transplant studies (Abrahamson et al. 1998; Gould and Dove 1996, 1997; Matsusaka et al. 1999; Zambrowicz et al. 1997).

We also reported that two independently derived lines of congenic ROSA26 mice carried a congenic interval of about 25 cM of 129-derived DNA flanking the *LacZ-neo<sup>R</sup>* insertion (Kohlhepp et al. 2000). Both lines of ROSA26 mice were also shown to carry the ROSA26-associated modifier. To map the modifier more precisely, we had generated four lines of mice that carried recombinant intervals within the ROSA26 interval. The single congenic line that carried the insertion site, Rec2, was the only line to show resistance to mammary tumor formation. This analysis mapped the resistance to a 4-cM interval that contains 129S7-derived flanking DNA and the *LacZ-neo<sup>R</sup>* insertion. Based on those studies, we could not determine whether the resistance was due to the insertion or to a tightly linked 129-derived modifier allele.

In this report, we provide evidence that the *LacZ-neo<sup>R</sup>* insertion is required for the resistance to mammary tumor development. We tested two additional congenic lines carrying the insertion and show that, regardless of the extent of congenic flanking DNA, the phenotype is similar. We used two approaches to address the question of whether the insertion is required for resistance. First, we characterized the tumor susceptibility of two congenic lines of B6 mice that carry 129-derived DNA in the modifier interval on Chr 6, but do not carry the *LacZ-neo<sup>R</sup>* insertion. Second, we generated a backcross between 129X1/SvJ (129X1) mice and ROSA26 mice and tested the backcross population for mammary tumor susceptibility. The results indicate that the resistance to mammary tumors in ROSA26 mice is due to the *LacZ-neo<sup>R</sup>* insertion itself and not to a tightly linked 129-derived modifier allele.

### Materials and methods

**Mice.** All mice were bred at the University of Wisconsin Medical School Animal Care Facility. The *Min* pedigree is maintained by backcrossing *Min/+* males to B6 females. The B6 *Min/+* parents for these experiments were from generations N42–N51. Animals were genotyped for *Min* by PCR by using an allele-specific PCR assay (Dietrich et al. 1993).

The Rec4 and Rec5 lines were derived from the B6.ROSA26 congenic line. Founders for each line were selected by identifying mice recombinant within the ROSA26 congenic interval on Chr 6. Mice heterozygous for the congenic interval will be designated Rec4/+ or Rec5/+. Rec4/+ and Rec5/+ mice were produced by crossing Rec4/+ or Rec5/+ males to B6 female mice. To assess tumor susceptibility, Rec4/+ or Rec5/+ females were crossed to B6.*Min/+* males, and the resulting *Min/+* female progeny were ENU treated. Rec4 and Rec5 mice were at generation N25–N27 at the time of testing.

Mice designated B6.129X1-*D6Mit36*-*D6Mit150* (Chr6-X1) were produced by crossing a 129X1/SvJ male mouse to a B6 female mouse. Males heterozygous for SSLP markers from *D6Mit36* to *D6Mit150* were selected each generation and backcrossed to B6 female mice. In generations N2–N4, we also selected for mice that were homozygous for B6 alleles in three

regions of the genome: *D2Mit7-D2Mit48* (59 cM), *D4Mit18-D4Mit33* (74 cM), and *D7Mit56-D7Mit44* (47 cM). To assess tumor susceptibility, Chr6-X1 (N5) females were crossed to *B6.Min/+* males, and the resulting *Min/+* female progeny were ENU treated.

B6.129P2-*D6Mit36-D6Mit150* (Chr6-P2) mice were derived from B6.129P2-*Tgfa<sup>tm1Ard</sup>* (*B6.Tgfa<sup>tm1Ard</sup>*) (N10F6) mice obtained from The Jackson Laboratory (Bar Harbor, ME). B6.*Tgfa<sup>tm1Ard</sup>* mice were found to retain 129-derived alleles at SSLP markers spanning the interval of *D6Mit3* to *D6Mit150*. *Tgfa* maps between *D6Mit3* and *D6Mit36* (Mann et al. 1993). A B6.*Tgfa<sup>tm1Ard</sup>* male mouse (N10F6N1) was crossed to a B6 female mouse, and a male offspring that no longer carried the 129 allele at *D6Mit3* or the *Tgfa<sup>tm1Ard</sup>* targeted allele, but retained 129 DNA in the interval from *D6Mit36* to *D6Mit150*, was identified. This mouse was crossed to B6 female mice to produce a line, designated Chr6-P2, segregating for the congenic chromosome. To assess tumor susceptibility, Chr6-P2 female mice that were heterozygous in the congenic interval were crossed to *B6.Min/+* male mice, and the resulting *Min/+* progeny were ENU treated.

F<sub>1</sub> parents for the ROSA backcross were produced by crossing B6.129S7-*Gro26* female mice (N19-N20) to a 129X1/SvJ male mouse. The resulting F<sub>1</sub> mice were of two genotypes with respect to the Chromosome 6 inherited from the B6.129S7-*Gro26* parent: those that had inherited the *LacZ-neo<sup>R</sup>* insertion, and those that had inherited the B6 chromosome and did not carry the insertion (Fig. 3). All mice carried the 129X1 Chr 6 derived from the male parent. Females of each of these genotypes were crossed to *B6.Min/+* males, and the resulting female *Min/+* backcross mice were ENU treated.

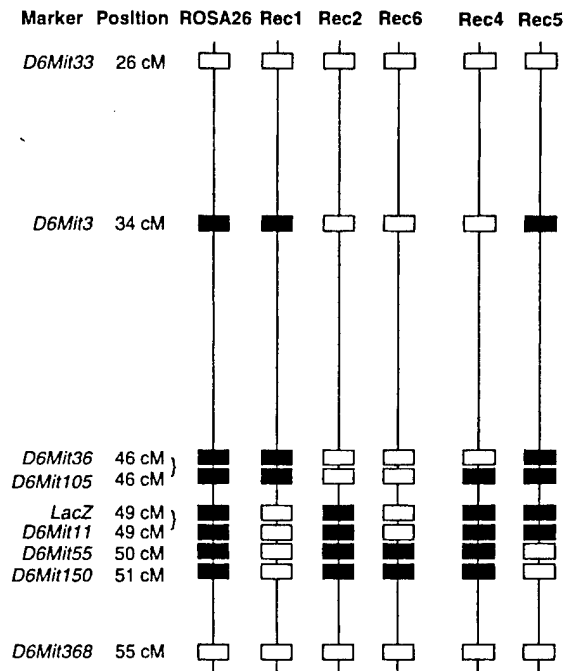
**ENU treatment and tumor scoring.** Mice were given a single i.p. injection of 50 mg/kg body weight ENU (Sigma Chemical, St. Louis, Mo.) at between 35 and 45 days of age (Moser et al. 1993). Mice were palpated weekly to detect mammary tumors. B6 *Min/+* mice from the *Min* colony were included in all rounds of mutagenesis as contemporaneous controls. Mice from the crosses to the Rec4, Rec5, Chr6-X1, and Chr6-P2 lines were sacrificed when moribund or 60 days after ENU treatment. Backcross mice were sacrificed when moribund or 100 days after ENU treatment. Mammary tumors were counted and collected at the time of sacrifice. Mammary tumors were identified as discrete masses generally larger than 3 mm in diameter. Mammary tumors were fixed in formalin for sectioning and histological analysis.

**PCR genotyping.** The presence of the ROSA26 insertion was confirmed by testing for the presence of *LacZ* as previously reported (Kohlhepp et al. 2000). Genotyping for SSLP markers on Chr 6 was done as previously described (Dietrich et al. 1992). Presence or absence of the *Tgfa* knockout was confirmed by using primers for *neoR*, 5'-AGGATCTCCTGTCATCTCACCTTGCTCCTG-3' and 5'-AAGAACTCGTCAAGAAGGCCGATAGAAGGCG-3'. PCR was run on an MJ Research PTC-100 Programmable thermal controller. The PCR conditions were: 1.25 mM MgCl<sub>2</sub>, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.2 mM dNTPs, 0.8 μM of each primer, 1.25 units of Promega Taq polymerase in storage buffer A, and genomic DNA for a total reaction volume of 25 μl. Initial denaturation at 92°C for 2 min, followed by 30 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 45 s, and extension at 72°C for 45 s, followed by extension at 72°C for 5 min. PCR products were electrophoresed in 2% agarose and visualized with EtBr staining.

**Statistical analysis.** Analyses were performed with the MSTAT computer program, provided by Norman Drinkwater at the McArdle Laboratory for Cancer Research. Two-sided *p* values were calculated for tumor multiplicity by using the Wilcoxon Rank Sum test. Two-sided *p* values were calculated to compare tumor incidence with Fisher's exact test.

## Results

**Rec4 and Rec5 mice are resistant to mammary tumor development.** As further confirmation that the region surrounding the *LacZ-neo<sup>R</sup>* insertion was required for tumor resistance, we generated and tested two additional recombinant congenic lines that carry the *LacZ-neo<sup>R</sup>* insertion, designated Rec4 and Rec5 (Fig. 1). Both of these lines were derived from the same B6.ROSA26 line as were the previous recombinants tested (Kohlhepp et al. 2000). Hetero-



**Fig. 1.** A representation of a portion of Chr 6 is shown for the ROSA26, Rec1, Rec2, Rec6 (Kohlhepp et al. 2000), Rec4, and Rec5 mice. The position from the centromere for each marker was obtained from Mouse Genome Database (MGD), Mouse Genome Informatics, The Jackson Laboratory, Bar Harbor, Maine. World Wide Web (URL: <http://www.informatics.jax.org/>) (8/01/2000). The order of the markers is the same as that obtained from MGD except for *D6Mit36* and *D6Mit105*. The most likely order is shown for *D6Mit36* and *D6Mit105* based on our recombinants. A filled box indicates a 129 allele at the locus; an open box indicates a B6 allele. For *LacZ*, a filled box indicates the presence of the insertion; an open box indicates the absence of the insertion.

zygous Rec4 or Rec5 mice were crossed to *B6.Min/+* mice, and the *Min/+* female progeny were ENU treated and followed to test for mammary tumor susceptibility. Rec4/+ *Min/+* mice developed significantly fewer mammary tumors than did their +/+ *Min/+* sibs or than did the *B6.Min/+* control mice (Table 1). In addition, significantly fewer Rec4/+ *Min/+* mice developed mammary tumors than either their +/+ *Min/+* sibs or the *B6.Min/+* control mice (Table 1). There was no significant difference in either mammary tumor multiplicity or incidence between the +/+ *Min/+* mice and the *B6.Min/+* control mice. Both the incidence and multiplicity of mammary tumors was also significantly different for the Rec5/+ *Min/+* mice compared with their +/+ *Min/+* sibs or with the *B6.Min/+* control mice (Table 1). Again, the +/+ *Min/+* mice did not differ from the *B6.Min/+* control animals with respect to either the multiplicity or incidence of mammary tumors. These results are consistent with our previous observation that the region between *D6Mit105* and *D6Mit55*, which includes the *LacZ-neo<sup>R</sup>* insertion, confers resistance to mammary tumor development in ENU-treated *Min/+* mice (Kohlhepp et al. 2000).

**Tumor resistance is not due to a linked modifier locus.** To test the hypothesis that the ROSA26-associated modifier effect is due to a 129-derived modifier allele within the *D6Mit105-D6Mit55* interval, we generated two lines of mice designated B6.129X1-*D6Mit36-D6Mit150* (Chr6-X1) and B6.129P2-*D6Mit36-D6Mit150* (Chr6-P2) (Fig. 2). Both lines carry 129-derived DNA from *D6Mit36* to *D6Mit150*, but neither line carries the ROSA26 *LacZ-neo<sup>R</sup>* insertion. If there were a modifier locus on Chr 6, we would expect to see a smaller number of mammary tumors in the mice carrying either congenic interval. Mice heterozygous for the interval from each line were crossed with *B6.Min/+* mice, and the

**Table 1.** The incidence and multiplicity of mammary tumors in ENU-treated congenic recombinant *Min/+* mice.

Line	Chr 6 Genotype <sup>a</sup>	Number of Mice	Number with Mammary Tumors (%)	Average Mammary Tumors ( $\pm$ SD)
Rec4	129/B6	14	6 (43) <sup>b</sup>	1.1 $\pm$ 1.4 <sup>c</sup>
	B6/B6	17	15 (88)	3.2 $\pm$ 1.9
B6	B6/B6	52	50 (96)	2.9 $\pm$ 1.9
Rec5	129/B6	16	5 (31) <sup>d</sup>	0.4 $\pm$ 0.6 <sup>e</sup>
	B6/B6	14	14 (100)	2.7 $\pm$ 1.8
B6	B6/B6	31	31 (100)	3.1 $\pm$ 2.1

<sup>a</sup> The mice that inherited the congenic chromosome are designated as 129/B6, while those that inherited the B6 chromosome are designated B6/B6. The B6 mice are B6 *Min/+* female mice treated with ENU at the same time as the congenic mice.

<sup>b</sup>  $P = 0.02$  compared with B6/B6 sibs and  $P = 2 \times 10^{-5}$  compared with B6 *Min/+* controls.

<sup>c</sup>  $P = 0.002$  compared with B6/B6 sibs and  $P = 7 \times 10^{-4}$  compared with B6 *Min/+* controls.

<sup>d</sup>  $P = 8 \times 10^{-5}$  compared with B6/B6 sibs and  $P = 2 \times 10^{-7}$  compared with B6 *Min/+* controls.

<sup>e</sup>  $P = 3 \times 10^{-5}$  compared with B6/B6 sibs and  $P = 2 \times 10^{-7}$  compared with B6 *Min/+* controls.

**Table 2.** The incidence and multiplicity of mammary tumors in ENU-treated Chr 6 congenic *Min/+* mice.

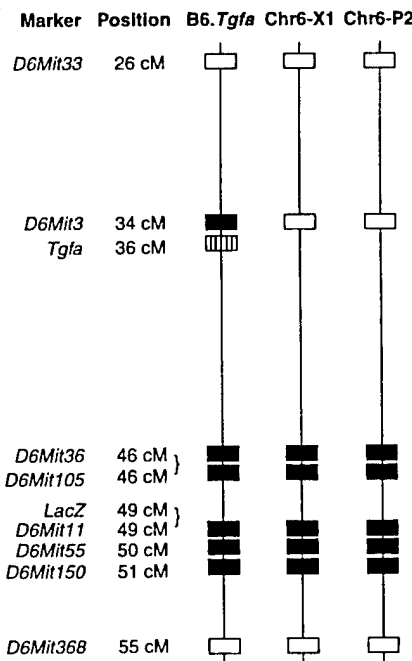
Line	Chr 6 Genotype <sup>a</sup>	Number of Mice	Number with Mammary Tumors (%)	Average Mammary Tumors ( $\pm$ SD)
Chr6-X1	129/B6	20	20 (100)	2.9 $\pm$ 1.7
	B6/B6	16	15 (94)	3.8 $\pm$ 2.4
B6	B6/B6	45	45 (100)	3.3 $\pm$ 1.8
Chr6-P2	129/B6	24	20 (83)	3.8 $\pm$ 2.5
	B6/B6	26	24 (92)	3.1 $\pm$ 1.9
B6	B6/B6	87	82 (94)	3.3 $\pm$ 2.0

<sup>a</sup> The mice that inherited the congenic chromosome are designated as 129/B6, while those that inherited the B6 chromosome are designated B6/B6. The B6 mice are B6 *Min/+* female mice treated with ENU at the same time as the congenic mice.

derived E14TG2a ES cells. B6.*Tgfa*<sup>tm1Ard</sup> mice carry 129 alleles at SSLP markers within the *D6Mit3* to *D6Mit150* interval. Thus, these mice retain a similar congenic region as do the B6.ROSA26 mice (Fig. 2). The *Tgfa* gene maps between *D6Mit3* and *D6Mit36* (Mann et al. 1993; Fig. 2). To eliminate any possible effect of heterozygosity for a mutant allele of *Tgfa*, we selected for a male mouse that did not carry the targeted allele of *Tgfa*, but did carry 129 alleles from *D6Mit36* through *D6Mit150*. This male founded the Chr6-P2 line. Female mice heterozygous for the Chr6-P2 congenic interval were crossed to B6 *Min/+* male mice, and the resulting *Min/+* female offspring were ENU treated and followed for tumor susceptibility. Chr6-P2 mice that were 129/B6 for the congenic interval did not differ from their sibs B6/B6 in the congenic region or the B6 *Min/+* control mice with respect to the incidence or multiplicity of mammary tumors (Table 2). Thus, these congenic mice, which carry 129-derived DNA in the region of the ROSA26-associated modifier, do not carry alleles that confer resistance to mammary tumors.

These experiments with two congenic lines carrying 129-derived alleles in the minimal modifier region provide no evidence for a 129 allele mapping to this region of Chr 6 that can confer resistance to mammary tumor development. Therefore, these results do not support the hypothesis that the ROSA26-associated modifier is due to a tightly linked 129-derived modifier allele. Thus, the resistance is most likely due to insertion.

*The LacZ-neoR insertion affects mammary tumor susceptibility in backcross mice.* As a second test of whether the *LacZ-neoR* insertion was necessary to confer resistance to mammary tumor development, we analyzed the effect of the insertion on tumor development in a set of backcross mice (Fig. 3). This backcross analysis served two purposes. First, it allowed us to test for a 129-derived modifier on Chr 6 without having to produce a congenic line. Second, it allowed us to assess the effect of the ROSA26-associated modifier in mice of a mixed genetic background. To produce the parents of the backcross mice, B6.ROSA26/+ female mice were crossed to a 129X1/SvJ male mouse (Fig. 3). The B6.ROSA26/+ mice used were heterozygous 129S7/B6 from *D6Mit3* to *D6Mit150* and carried the *LacZ-neoR* insertion. The F<sub>1</sub> offspring from this cross consisted of mice of two genotypes with respect to Chr 6: those that carried the 129S7/ROSA26 congenic interval across from a 129X1 Chr 6 and those that carried a B6 Chr 6 across from a 129X1 Chr (Fig. 3). At all other loci, these mice should have been heterozygous B6/129X1. F<sub>1</sub> females of each of these Chr 6 genotypes were crossed to B6 *Min/+* males and the resulting (B6/129X1F<sub>1</sub>)B6 female *Min/+* backcross progeny were treated with ENU and followed for tumor susceptibility. The backcross population analyzed consisted of mice of three genotypes with respect to Chr 6: those heterozygous 129X1/B6 from *D6Mit3* to *D6Mit150* (designated Chr6-129X1), those heterozygous 129S7/B6 from *D6Mit3* to *D6Mit150* and carrying the ROSA26 insertion (designated Chr6-ROSA), and those B6/B6 from *D6Mit3* to *D6Mit150* (designated Chr6-B6; Fig. 3). Mice that were recom-

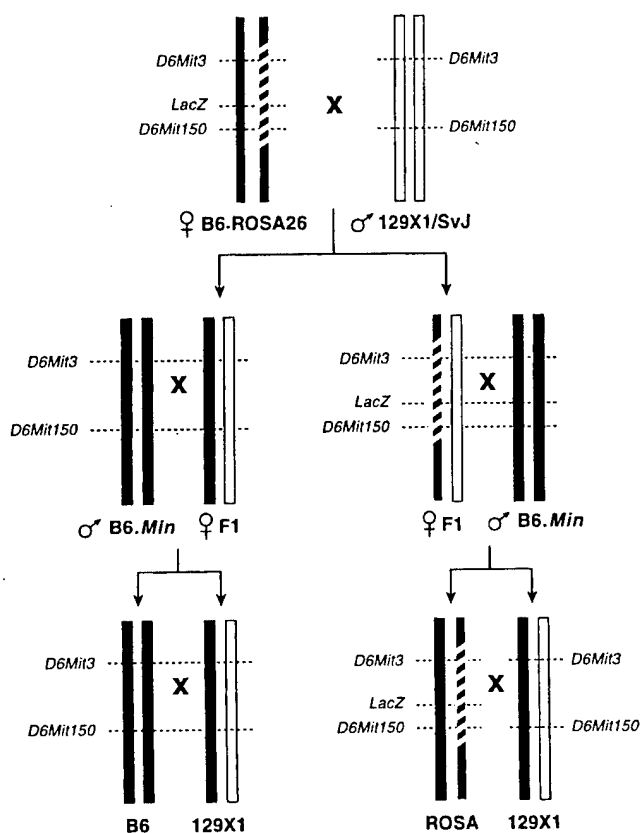


**Fig. 2.** A representation of a portion of Chr 6 is shown for the *Tgfa*<sup>tm1Ard</sup> (B6.*Tgfa*), Chr6-X1, and Chr6-P2 mice. The position from the centromere for the *Tgfa* gene and each SSLP marker was obtained from Mouse Genome Database (MGD), Mouse Genome Informatics, The Jackson Laboratory, World Wide Web (URL: <http://www.informatics.jax.org/>) (8/01/2000). A filled box indicates a 129 allele at the locus; an open box indicates a B6 allele; and a hatched box the *Tgfa*<sup>tm1Ard</sup> allele.

resulting *Min/+* female progeny were treated with ENU and scored for mammary tumors.

The Chr6-X1 line carries 129 alleles derived from the 129X1/SvJ strain, and was at the sixth backcross generation at the time of testing. Chr6-X1 *Min/+* mice 129/B6 for the congenic region were as susceptible to mammary tumors as were the Chr6-X1 *Min/+* mice B6/B6 in the region or as were the B6 *Min/+* control mice (Table 2). Therefore, the mice carrying 129 alleles in the *D6Mit36*–*D6Mit150* interval do not carry a modifier allele that results in increased resistance to mammary tumor development.

The Chr6-P2 mice provide a second test of the effect of 129 alleles in this region on tumor development. These mice were derived from B6.129P2-*Tgfa*<sup>tm1Ard</sup> (B6.*Tgfa*<sup>tm1Ard</sup>) mice (Mann et al. 1993). The *Tgfa*<sup>tm1Ard</sup> targeted mutation was made using 129P2



**Fig. 3.** A representation of the ROSA backcross is shown. For each generation, only Chr 6 is shown. A solid bar represents B6 DNA, an open bar represents 129X1 DNA, and a hatched bar represents 129S7 DNA from the ROSA26 parent. In the backcross progeny, only the non-recombinant chromosomes are shown. Any mice recombinant between *D6Mit3* and *D6Mit150* were not included in the study. Recombination events may have occurred proximal to *D6Mit3* and distal to *D6Mit150*.

binant in the *D6Mit3* to *D6Mit150* interval were excluded from the analysis. Animals of all three Chr 6 genotypes should have been segregating randomly for 129 and B6 alleles at loci on all other chromosomes. Thus, these backcross mice allow us to test for an effect of Chr 6 alleles on mammary tumor susceptibility.

The average mammary tumor number was reduced more than twofold in the backcross mice relative to the B6 *Min/+* controls (Table 3). To determine whether genes controlling mammary tumor susceptibility mapped to Chr 6, and more specifically to the ROSA26 Chr 6, we separated the backcross population by the three Chr 6 genotypes, Chr6-ROSA, Chr6-129X1, and Chr6-B6, and analyzed tumor numbers. The Chr6-ROSA backcross mice had significantly fewer mammary tumors than did the Chr6-129X1 backcross mice, the Chr6-B6 backcross mice, or the control B6 *Min/+* mice (Table 3). Chr6-ROSA mice developed at least twofold fewer mammary tumors than did the Chr6-129X1 mice or the Chr6-B6 mice and approximately sixfold fewer tumors than did the control B6 *Min/+* mice. This indicates that the ROSA26 Chr 6 carries at least one modifier allele not present on the 129X1 Chr 6. With respect to mammary tumor incidence, the Chr6-ROSA mice were significantly less susceptible than the control B6 *Min/+* mice, but were not significantly different from the Chr6-129X1 mice or the Chr6-B6 mice.

Both the Chr6-129X1 and Chr6-B6 backcross mice also developed approximately twofold fewer mammary tumors than did the control B6 *Min/+* mice, a significant difference. In addition, fewer Chr6-129X1 and Chr6-B6 mice developed mammary tumors compared with the control B6 *Min/+* mice. Thus, the 129X1 strain must carry alleles that can modify susceptibility to mam-

**Table 3.** The incidence and multiplicity of mammary tumors in ENU-treated ROSA Backcross *Min/+* mice.

Line	Chr 6 Genotype <sup>a</sup>	Number of Mice	Number with Mammary Tumors (%)	Average Mammary Tumors ( $\pm$ SD)
RBC	All	130	70 (54) <sup>b</sup>	1.1 $\pm$ 1.4 <sup>c</sup>
RBC	ROSA	31	12 (39) <sup>d</sup>	0.5 $\pm$ 0.8 <sup>e</sup>
	129X1	83	48 (58) <sup>f</sup>	1.2 $\pm$ 1.4 <sup>g</sup>
	B6	16	10 (62) <sup>h</sup>	1.6 $\pm$ 1.7 <sup>i</sup>
B6	B6	163	150 (92)	2.9 $\pm$ 1.8

<sup>a</sup> Mice designated ROSA are heterozygous 129/B6 from *D6Mit3* and *D6Mit150* and carry the ROSA26 insertion. Mice designated 129/B6 are heterozygous 129/B6 from *D6Mit3* and *D6Mit150* and do not carry the insertion. The mice designated B6 are homozygous B6 from *D6Mit3* and *D6Mit150*. The B6 mice are B6 *Min/+* female mice treated with ENU at the same time as the congenic mice. Mice that were recombinant between *D6Mit3* and *D6Mit150* were not included in the study.

<sup>b</sup>  $P = 5 \times 10^{-14}$  compared with B6 *Min/+* controls.

<sup>c</sup>  $P = 2 \times 10^{-18}$  compared with B6 *Min/+* controls.

<sup>d</sup>  $P = 2 \times 10^{-10}$  compared with B6 *Min/+* controls.

<sup>e</sup>  $P = 0.02$  compared with 129X1,  $P = 0.03$  compared with B6, and  $P = 6 \times 10^{-12}$  compared with B6 *Min/+* controls.

<sup>f</sup>  $P = 7 \times 10^{-10}$  compared with B6 *Min/+* controls.

<sup>g</sup>  $P = 1 \times 10^{-12}$  compared with B6 *Min/+* controls.

<sup>h</sup>  $P = 0.003$  compared with B6 *Min/+* controls.

<sup>i</sup>  $P = 0.007$  compared with B6 *Min/+* controls.

mary tumor development in *Min/+* mice. However, the Chr6-129X1 mice were not different from the Chr6-B6 mice with respect to mammary tumor number or incidence. If there were a 129-derived modifier on Chr 6 conferring mammary tumor resistance, we would have expected the Chr6-129X1 mice, which are 129/B6 from *D6Mit3* to *D6Mit150*, to be more resistant than the Chr6-B6 mice, which are B6/B6 from *D6Mit3* to *D6Mit150*. Therefore, the modifier loci carried by the 129X1 strain do not map to Chr 6. Thus, this experiment fails to provide support for the hypothesis that the resistance seen in the ROSA26 mice is due to a linked 129-derived modifier allele. It does provide evidence that the ROSA26 insertion is required for the resistant phenotype.

## Discussion

We have previously reported that two congenic lines of B6.ROSA26 mice, R26-1 and R26-2 (Kohlhepp et al. 2000), both carried alleles that conferred resistance to mammary tumor development after ENU treatment. In an attempt to map the loci responsible for the resistance, we had analyzed several lines that carried smaller portions of the congenic interval. We identified a single congenic recombinant, Rec2, that carried a smaller congenic interval that included the insertion and reported that *Min/+* mice carrying the Rec2 interval were also resistant to mammary tumor development. Three congenic recombinant lines that did not carry the insertion were sensitive to mammary tumor development. To confirm that the insertion was required for resistance, we generated two additional congenic recombinant lines that carry the *LacZ-neo<sup>R</sup>* insertion. Here we report that mice that carry either the Rec4 or the Rec5 intervals, both of which include the insertion, are also resistant to mammary tumor development. Thus, we have identified three recombinant lines derived from the B6.ROSA26 line that carry the insertion and are resistant to mammary tumor development. Although the Rec4 and Rec5 lines do not make the interval containing the modifier smaller than previously reported, they do confirm that the modifier maps to the 4-cM interval from *D6Mit105* to *D6Mit55* that contains the *LacZ-neo<sup>R</sup>* insertion. Additionally, the similarity of the phenotype of the ROSA26/+ mice from each of the recombinant lines indicates that the modifier locus maps to the *D6Mit105* to *D6Mit55* interval.

On the basis of the analysis of the recombinants, we could not determine whether the resistance was caused by the insertion or a tightly linked 129-derived modifier allele. We took two approaches to test for a 129-derived modifier allele within the inter-

val. One approach was to test mice that were congenic (or nearly congenic) for 129-derived DNA within the ROSA26 interval for the presence of a modifier. B6.ROSA26 mice were derived from AB1 129 ES cells (Soriano et al. 1991), which were derived from 129S7/SvEvBrd-Hprt<sup>b-m2</sup> (129S7) mice. Because 129S7 mice were not readily available, we tested for the presence of a modifier on Chr 6 by using congenic mice derived from two other 129 strains. Chr6-X1 mice were derived from the 129X1/SvJ strain, and Chr6-P2 mice were derived from the 129P2/OlaHsd strain via the E14TG2a ES cell line. We used mice from two diverse 129 strains, in case there were some genetic differences, although Chr 6 is not a region where the 129 strains are known to differ (Simpson et al. 1997). In addition to the published information, we have tested approximately 50 SSLP markers on Chr 6 and have found no polymorphisms between 129X1, 129S6/SvEvTac (closely related to 129S7), and ROSA26 mice (data not shown). Thus, there is reason to expect that these two congenic lines of mice provide a good test for the presence of a 129-derived modifier allele in this region. However, no evidence for a 129-derived modifier of mammary tumor development within the minimal modifier interval was obtained from analysis of these congenic mice.

The second approach was to test for an effect on mammary tumor development in backcross mice segregating for Chr 6 carrying or not carrying the insertion. The backcross analysis provided more evidence for the effect of the *LacZ-neo<sup>R</sup>* insertion on mammary tumor susceptibility. The twofold reduction in tumor number in mice carrying the insertion compared with the backcross mice carrying 129-derived DNA in the region, but not the insertion, is indicative of the effect of the insertion. This experiment also provides evidence that the 129X1 strain carries dominantly acting alleles at mammary modifier loci. However, there is no evidence for a modifier locus that maps to Chr 6. In the (B6.ROSA26/129X1)B6 backcross population, where mammary tumor number was already decreased approximately twofold compared with B6 *Min/+* controls, the ROSA26 insertion results in a further twofold reduction in tumor numbers. Thus, the effect of the ROSA26 insertion at least is additive with the effect of the unmapped modifier loci segregating in the backcross.

The results of these experiments strongly support the hypothesis that the tumor resistance seen in ROSA26 mice is due to the *LacZ-neo<sup>R</sup>* insertion. However, they do not provide a mechanism for how the insertion results in tumor resistance in mice heterozygous for the insertion. Examination of the insertion site provides no ready explanation for the effect on tumor development. The *LacZ-neo<sup>R</sup>* reporter gene is inserted into a region that produces three transcripts (Zambrowicz et al. 1997). Two transcripts, transcript 1 and 2, share a promoter and the first exon and have no open reading frames. The third transcript, transcript AS, originates from the reverse strand and potentially encodes a novel 505 amino acid protein of unknown function. The functions of transcripts 1 and 2 are unknown, but as transcript 2 overlaps transcript AS, it may normally function as an antisense regulator of transcript AS. In ROSA26 mice, the retroviral insertion is in the intron between exon 1 and 2 of transcript 1 and 2. The transcript that encodes the *LacZ-neo<sup>R</sup>* uses the shared promoter and includes the first shared exon. The insertion disrupts the expression of both transcripts 1 and 2 in mice homozygous for the ROSA26 insertion. Transcript AS is expressed in ROSA26 homozygous mice in multiple tissues at apparently normal levels. However, it is possible that transcript 2 is involved in regulating the levels of transcript AS. The expression of each of these transcripts remains to be tested in the mammary gland and in mice heterozygous for the insertion.

The ROSA26 insertion also results in the ubiquitous expression of a fusion protein with  $\beta$ -galactosidase and neomycin phosphotransferase activities. Our results do not allow us to rule out the expression of this fusion protein as the mechanism of tumor resistance in ROSA26 mice. The neomycin resistance gene encodes an aminoglycoside resistance enzyme. Other aminoglycoside bac-

terial resistance enzymes have been shown to have homology to eukaryotic protein kinases (Hon et al. 1997) and have been shown to be inhibited by eukaryotic protein kinase inhibitors (Daigle et al. 1997). Thus, this bacterial enzyme has the potential to act as a protein kinase in a mammalian system, especially when expressed in high levels as it is in the ROSA26 mice. The role of expression of this fusion protein in tumor development can be tested in mice with a modified ROSA26 locus, which has been engineered to contain a floxed stopper fragment (Mao 1999). These mice do not express the fusion protein unless the stopper fragment is removed by Cre recombinase. However, the insertion does result in the elimination of transcripts 1 and 2.

The insertion may also disrupt the expression of neighboring genes. This region on mouse Chr 6 is homologous to human Chr 3p25, which has been found to be a frequent target of LOH in human breast tumors (Matsumoto et al. 1997). This suggests that at least one gene within this region can affect mammary tumor development. The VHL (Von Hippel-Lindau) locus maps to human 3p25 and in the mouse between *D6Mit11* and *D6Mit55*, placing it within the minimal modifier region (Street et al. 1998). However, it does not frequently have point mutations in breast cancers (Gnarra et al. 1994) and is not within the most commonly deleted region in breast cancers (Matsumoto et al. 1997). At present, there are no other obvious candidate genes mapped to this region.

The magnitude of the effect of the insertion in all cases is at least a twofold reduction in the number of mammary tumors. In all of these experiments, the mice tested were heterozygous for the insertion; thus, the effect is at least semidominant. Owing to difficulty in producing mice homozygous for the insertion, we have been unable to test for the mammary tumor phenotype of such mice. Thus, it is difficult to predict whether the effect of the insertion will result from loss of function or gain of function. Loss of function could result from the loss of expression of the two noncoding transcripts caused by the insertion or repression of transcription of nearby genes. Several reports have recently described the effects of insertions on the expression of nearby loci. In particular, in two cases involving the insertion of a retroviral sequence encoding a *LacZ-neo<sup>R</sup>* fusion product, the *Gtlac* and *Etlac* insertions are reported to result in decreased expression of a nearby gene (Schmidt et al. 1997; Schuster-Gessler et al. 1996; Zachgo et al. 1998). If the effect were due to loss of function, it would be expected that mice homozygous for the insertion would be even more resistant to tumor development. Gain of function could result from the dysregulation of nearby loci, from the loss of regulation owing to a decreased level of transcripts 1 and 2, or from the expression of the  $\beta$ -galactosidase-neomycin resistance fusion protein. Alternatively, the effect may be due to more than one of these mechanisms. A full understanding of the effect of this insertion on tumor development will require thorough investigation of each of these possibilities.

Although the mechanism is unknown, the ROSA26 insertion has a strong effect on mammary tumor development in *Min/+* mice. Unlike tumor susceptibility or modifier loci identified through backcross analysis, the insertion site in the ROSA26 mice provides a starting point for the molecular characterization of this modifier. This information, in combination with sequence data from both the human and mouse genome project, should provide possible candidates for testing. The availability of multiple congenic lines will also make further biological characterization of the modifier possible.

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